INFORMATION NETWORKS IN THE MAMMARY GLAND

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Abstract | Unique developmental features during puberty, pregnancy, lactation and post-lactation make the mammary gland a prime object to explore genetic circuits that control the specification, proliferation, differentiation, survival and death of cells. Steroids and simple peptide hormones initiate and carry out complex developmental programmes, and reverse genetics has been used to define the underlying mechanistic connections.

MAMMARY GLAND
A secretory organ in mammals that produces milk during lactation to feed the young. The gland is composed of alveoli, ducts and a stromal compartment.

LIPID RAFTS
Lateral aggregates of cholesterol and sphingomyelin that are thought to occur in the plasma membrane.

GAP JUNCTION
Communicating junction (permeant to molecules up to 1 kDa) between adjacent cells, which is composed of 12 connexin protein subunits, six of which form a connexon or hemichannel contributed by each of the coupled cells.

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Organogenesis requires a sequence of cellular processes that involve commitment to a specific cell fate, proliferation of committed progenitor cells, the initiation and implementation of differentiation programmes and the maintenance of tissue homeostasis by the controlled turnover of cells. In the MAMMARY GLAND, these events are strictly regulated by steroid and peptide hormones. A number of reviews cover this subject in depth[1–4]. Over the past two decades, studies with numerous strains of genetically altered mice have resulted in the characterization of an integrated framework of signalling networks that control the normal development (BOX 1) and neoplastic conversion of mammary tissue. Concepts have emerged as to how mammary cells have acquired ancient signalling pathways and tailored them to satisfy the diverse needs of the developing tissue during puberty, pregnancy, lactation and regression of the gland in involution. In particular, it has become clear that various cytokine receptors signal through a limited set of tyrosine kinases and transcription factors. Recent studies have also identified 'brakes' that stop the flow of information emanating from the cell-surface receptors. These include molecules from the suppressor of cytokine signalling (SOCS) family and, possibly more unexpectedly, LIPID RAFTS. The role of structural components such as GAP JUNCTIONS has long been underappreciated but, as it turns out, such components are also crucial in mammary cell differentiation.

Tissue culture cells, primary cells and genetically altered mice have been used over the past two decades to dissect pathways that control mammary development and cellular differentiation. Although the use of primary cultures and tissue culture cell lines has shed light on the mechanistic cues that underlie signalling pathways, this approach could not be expected to recapitulate the developing architecture and physiological intricacies that occur during pregnancy, and final confirmation still relies on the use of animal models. More than 100 genes have been shown to control various aspects of mammary physiology, from the development of the mammary ANLAGE to remodelling of the gland during involution. Whereas some of these genes fit into pathways that can mechanistically explain their function, the position of others in signalling networks remains to be determined. Owing to space restrictions, this review focuses on the signalling pathways that function during pregnancy. This developmental window has been studied most intensively and a picture is emerging that links hormones to specific molecules that carry out their function.

Two genetic approaches have been used to explore proteins that control mammary development: the ectopic expression of genes in transgenic animals and the inactivation of genes from the mouse genome. In the first approach researchers ask which cellular events can be modulated by a given protein, whereas the second approach directly addresses the function of a protein *in vivo*. This review was written according to the motto, 'don't ask what a protein can do for a cell but ask what a cell can or cannot do without it'.

Mammary structure and cellular composition

Two tissue compartments constitute the mammary gland: the epithelium, which consists of DUCTS and

Box 1 | **Life and death of a mammary gland**

The mammary gland forms as an appendage of the skin and has its evolutionary origin in skin glands. The number and location of glands vary among different classes of mammals. In mice, five pairs of glands develop along a line that runs slightly ventral to the limb buds, whereas only one pair develops in the thoracic region in humans. Development of the mammary gland commences in the foetus. The initial cues that induce the formation of small buds on the ventral surface of foetuses are not known. Sequential and reciprocal signals between the epithelium and surrounding mesenchyme (the embryonic stroma) direct the outgrowth of a small duct into deeper layers of the dermal mesenchyme and formation of the nipple, the opening for milk removal. Through further elongation and bifurcation a small ductal system forms that associates with the subdermal fat pad. During puberty the cyclical production of ovarian oestrogen and progesterone accelerates ductal outgrowth and branching. In the mature animal, the entire fat pad is filled with a regularly spaced system of primary and secondary ducts that are decorated with SIDE BRANCHES, which form and disappear during each oestrous cycle. Proliferation and maturation of the alveolar compartment occurs during pregnancy and is controlled mainly by prolactin and PLACENTAL LACTOGENS. At term, the mammary gland reaches maturity and produces and secretes milk to support the young. In mice, mammary tissue produces milk equivalent to 20% of the body weight of the dam. At the end of lactation, the loss of suckling stimuli and the pressure build-up on cessation of milk removal initiates a remodelling programme called involution. This causes massive cell death, the collapse of the alveoli and the remodelling of the epithelial compartment to restore a simple ductal structure again. A new round of alveolar expansion, maturation and lactation is initiated with the next pregnancy.

milk-producing alveolar cells; and the STROMA, or connective tissue, which is also called the mammary fat pad (FIG. 1; BOX 1). In general, the epithelial cells form ducts and ALVEOLI with a central lumen that opens to the body surface through the nipple. By contrast, monotremes have mammary glands without a nipple or central lumen and the ducts open directly to a confined area known as the milk patch. Most epithelial cells are LUMINAL, SECRETORY CELLS, which undergo functional differentiation in pregnancy to produce milk. They are encased by a mesh-like system of BASAL, MYOEPITHELIAL CELLS, which are contractile and participate in the delivery of milk. The extensive system of ducts and alveoli is embedded in the stroma, the main components of which are ADIPOCYTES, but fibroblasts, cells of the haematopoietic system, blood vessels and neurons are also present.

The existence of distinct cell lineages that are derived from an elusive mammary STEM CELL has been proposed and models of how these lineages develop have been defined[5–7]. It has been proposed that the cells forming the epithelial compartment of the mammary gland are derived from mammary stem cells (MSCs), which have the capacity to self-renew and give rise to committed epithelial precursor cells (EPCs; FIG. 2). The progeny of EPCs then becomes restricted to a ductal or alveolar fate. The ductal precursor cells (DPs) form basal cells and luminal cells, the two cell types that constitute ducts. During pregnancy, alveoli are generated from alveolar precursors (APs), which give rise to basal and luminal cells — the differentiated, milk-producing cells. The alveolar epithelium expands during pregnancy, secretes milk during lactation and undergoes apoptosis and remodelling during involution. Loss of the prolactin signal after suckling is stopped leads to massive death of luminal cells in a process called involution. This restores a ductal system that contains multipotent and committed ductal and luminal precursor cells (FIG. 2). The presence of stem cells is the basis of the profound capacity for alveolar renewal in each subsequent pregnancy. Stem cells have the capacity to renew themselves and also give rise to progenitor cells, which are destined for either a basal or a luminal fate. The ability of progenitor cells to function as a source for alveolar development was highlighted by experiments in which small sections of a duct generated an entire ductal tree when transplanted into a cleared fat pad[8–10] (BOX 2).

ANLAGE
The embryonic primordium of an organ.

MAMMARY DUCT
An epithelial structure transporting milk.

SIDE BRANCHES
Small ducts that branch off a major duct during ductal elongation.

PLACENTAL LACTOGENS
Peptide hormones produced by the placenta during pregnancy. These hormones bind to the prolactin receptor and are involved in the induction of proliferation and functional differentiation of luminal cells.

MAMMARY STROMA
Connective tissue supporting the epithelial compartment of the mammary gland. The prevalent cell types are fat cells (adipocytes) in addition to fibroblasts, vasculature and haematopoietic cells.

ALVEOLI
Ball-shaped structures composed of two cell types surrounding a central lumen. The luminal cells synthesize and secrete milk components and the basal cells are contractile.

Postnatal development

Growing ducts in pubescent animals have conspicuous, club-shaped structures at their tips, which are known as terminal end buds (TEB; FIG. 1a). These are sites at which cells divide at a high rate to advance progression of the ducts into the fat pad[11]. They disappear once the entire fat pad has been filled with ducts (FIG. 1b). Two morphologically distinct cell types can be found in the TEBs — an outer layer of cap cells and the more centrally located body cells. The former gives rise to basal cells whereas luminal cells are derived from body cells.

In postnatal mammary tissue, most epithelial cells express receptors for oestrogen and progesterone to enable these hormones to stimulate ductal outgrowth and branching. Part of the action of oestrogen derives from the induction of progesterone receptors (PRs). In the pubescent gland, PRs are expressed in proliferating cells[12], and in virgin mice (female mice that have not been mated) that are treated with high doses of oestrogen and progesterone (to mimic pregnancy), proliferating cells are preferentially localized adjacent to PR-positive cells[13]. Both oestrogen and progesterone have pleiotropic actions in the uterus, ovaries and the hypothalamic–pituitary axis in regulating sexual development. In the mammary gland, oestrogen and progesterone control ductal outgrowth and alveolar expansion, respectively, namely by regulating cell proliferation and cellular turnover in the oestrous cycle.

Oestrogen. Oestrogen binds to two distinct receptors, oestrogen receptor (ER) α and ERβ, which are encoded by two genes. Like other steroid receptors, these are members of the large family of nuclear receptors, which function as transcription factors when bound to the steroid hormone. Whereas deletion of ERβ has no adverse effects on ductal and alveolar development[14], both stromal and epithelial ERα are required for normal ductal elongation and outgrowth during puberty[15]. By contrast, ERα is dispensable for pregnancy-mediated alveolar expansion[16].

Progesterone. There are also two isoforms — A and B — of the PR, which display differential activities as transcription factors. These isoforms are encoded by two transcripts derived from the same gene by

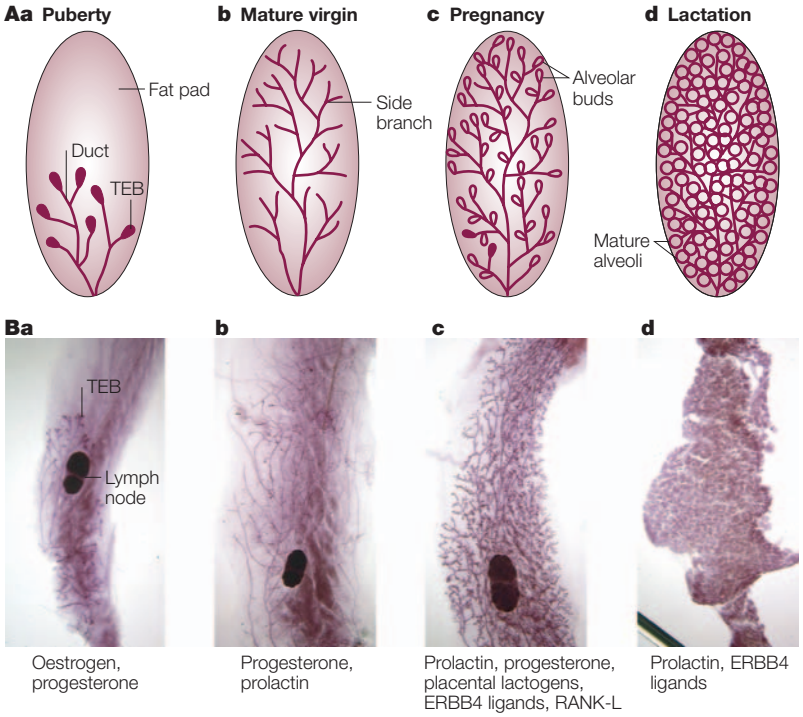


Figure 1 | **Mouse mammary gland development during puberty, pregnancy and lactation.** Schematic (**Aa**–**d**) and wholemount (**Ba**–**d**) presentation of the different stages and the principal hormones that control development. A rudimentary ductal design within the mammary fat pad is visible at birth, which grows at the same rate as the animal until the onset of puberty. During puberty, the cyclical production of ovarian oestrogen and progesterone promotes and accelerates ductal outgrowth (**Aa**, **Ba**). At this stage, conspicuous club-shaped structures (terminal end buds (TEB)), where the highest levels of cell division occur, appear at the ductal tips. In the mature virgin, the entire fat pad is filled with a regularly spaced system of primary and secondary ducts, with side branches that form and disappear in each oestrous cycle (**Ab**, **Bb**). Hormonal changes that occur when pregnancy begins (the release of prolactin, placental lactogens and progesterone) increase cell proliferation and the formation of alveolar buds (**Ac**, **Bc**), which grow and differentiate into milk-secreting alveoli at the end of pregnancy (**Ad**, **Bd**). During lactation, alveoli are fully matured and the luminal cells synthesize and secrete milk components into the lumina. RANK-L, receptor activator of nuclear factor κB (NF-κB)-ligand.

differential initiation of transcription. Alveolar development is perturbed in the combined absence of both isoforms[17], but there is no adverse effect on development if only PR-A is deleted[18], indicating that the PR-B form is required to carry out the proliferative effects of progesterone on mammary epithelial cells[19]. It should be noted that the PR is essential for the expansion of the alveolar compartment, but its contribution to ductal elongation and branching is only minor. PR-expressing cells are evenly spaced in the ducts of young mice and their distribution becomes mosaic in mature virgin mice and during early pregnancy[20,21]. In early pregnancy, PR-positive cells are found closely apposed to proliferating cells, which implies that the proliferative effect of PR is mediated in part through PARACRINE activities, as shown by mixing experiments. PR-negative cells, which by themselves cannot develop into functional alveoli, can participate in the development of alveoli when they are in close proximity to wild-type cells[22]. Progesterone seems to induce the production of a signal that induces the proliferation of neighbouring cells. One candidate for this activity is receptor activator of nuclear factor κB (NF-κB)-ligand (RANK-L, formerly called osteoprotegerin)[19], a molecule that belongs to the tumour necrosis factor (TNF) family and is an important regulator of OSTEOCLAST development[23].

LUMINAL, SECRETORY CELLS
Cells lining the lumen of alveoli. They synthesize milk proteins and secrete milk.

BASAL, MYOEPITHELIAL CELLS
Cells that surround the luminal cells as a layer. They contain smooth muscle actin and contract in response to oxytocin to mediate milk let-down through the main ducts to the nipple.

ADIPOCYTE
A fat cell.

STEM CELLS
Cells that have the capacity for self renewal and generation of differentiating daughter cells.

Processing information during pregnancy

Three characteristic and temporally coordinated cellular events, which are regulated by hormones, occur during pregnancy. These are the proliferation of alveolar epithelium, its differentiation and its survival. This developmental programme can be initiated and carried out by a single peptide hormone, prolactin (PRL), which is produced mainly by the LACTOTROPHS in the anterior pituitary gland. Over the past decade, several other CYTOKINES, including RANK-L[24] and ligands of the epidermal growth factor (EGF) family[25], have joined the ranks of 'inducers of mammary development'. The transcription factors signal transducer and activator of transcription-5a (STAT5A) and STAT5B (referred to collectively as STAT5) are shared downstream mediators of peptide hormones that signal through the prolactin receptor (PRLR) and ERBB4, and they are the central switch controlling proliferation, differentiation and survival of mammary cells (see below). The activation and nuclear localization of STAT5 and the expression of genes that encode milk proteins define the differentiation programme during pregnancy (FIG. 3).

Prolactin and placental lactogens. In 1929, Gruyter and Strijker injected pituitary extract from lactating rabbits into virgin rabbits and showed that this undefined mixture could induce mammary development and lactation (for historical references, see REF. 3). Four years later, Riddle and his colleagues identified the active component and named it 'prolactin' after its function in promoting lactation. Prolactin has two essential roles in reproduction — the maintenance of the corpus luteum during early pregnancy and the induction of mammary development. Through the maintenance of the CORPUS LUTEUM, PRL ensures the secretion of oestrogen and progesterone, which themselves are required for ductal and alveolar development, respectively (as outlined above). After mid-pregnancy, both functions of PRL are replaced by PLACENTAL LACTOGENS, but PRL takes over again after birth. PRL is essential for maintaining lactation, as evidenced by lactational failure after blocking PRL secretion with dopamine antagonists such as bromocriptine. Although the main source of PRL is the lactotrophs, local production of PRL by mammary epithelium has been reported[26], where it functions as a paracrine mediator of mammary epithelial development[27].

PRL mediates its action through PRLR, a transmembrane protein of the class I cytokine receptor family[28]. PRLR dimers undergo a conformational change following ligand binding, and the associated Janus kinase-2 (JAK2) phosphorylates specific tyrosine residues in the PRLR, which allows docking and

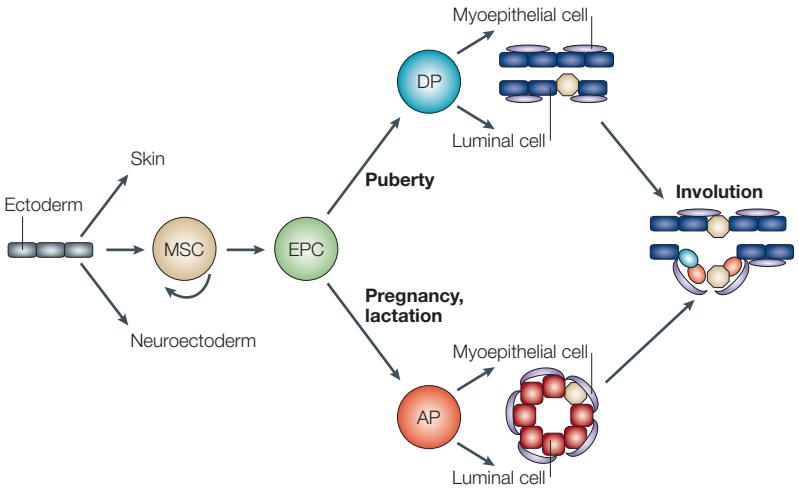


Figure 2 | **Cell lineages in mammary epithelium.** The mammary gland is a derivative of the ectoderm, which also gives rise to the skin and other appendages as well as the neuroectoderm. Models, supported by experimental evidence, have been developed that propose the existence of a mammary stem cell (MSC) and distinct cell lineages that lead to the formation of different cell types in mammary tissue[5]. It has been suggested that the multipotent MSCs give rise to epithelial precursor cells (EPCs), the progeny of which develop into either ductal or alveolar cells. Myoepithelial cells and luminal cells are formed from ductal precursors (DP) as the ducts grow out postnatally, particularly during puberty. On initiation of pregnancy, alveolar precursor cells (AP) give rise to myoepithelial and luminal cells, the latter of which synthesize and secrete milk. After lactation, the alveolar cells are subject to programmed cell death during the process of involution. A simple ductal system containing multipotent (yellow) and committed ductal (green) and luminal (orange) precursor cells persists that will develop into a fully functional epithelium in subsequent pregnancies.

activation of STAT5. As well as STAT5, PRLR can signal through the mitogen-activated protein kinase (MAPK) pathway and others that are dependent on JAK2. In addition to the full-length form (the long form), other isoforms of the PRLR have been detected. These include a short form that lacks the cytoplasmic domain except for 23 amino acids (see below).

Inactivation of the genes encoding PRL and its receptor, and the expression of transgenes encoding mutant PRLR, have helped to elucidate the role of this cytokine — at least during early stages of pregnancy. Female mice with inactive *PrlR* alleles had non-functional corpora lutea and so could not maintain pregnancies[29]. This, however, could be partially overcome with progesterone treatment. Although ductal outgrowth during puberty was overtly normal in these mice, no functional alveolar compartment was formed during pregnancy, as a result of a lack of luminal cell proliferation[29]. Transplantation experiments of mutant mammary tissue into wild-type hosts (BOX 2) established that the proliferation and differentiation defect was autonomous to the epithelial compartment and not the result of altered systemic hormone levels[30,31].

A threshold level of PRLR is required for normal development, as evidenced by a genetic HAPLO-INSUFFICIENCY[29]. In the presence of only one *PrlR* gene, alveolar proliferation and differentiation was stalled in the second half of pregnancy. This developmental block was partially alleviated after several pregnancies[29,31]. Kelly and colleagues explored the molecular basis of this by studying the expression of a transgene encoding a short version of PRLR that lacks the cytoplasmic domain except for 23 amino acids[32]. Expression of this transgene restored mammary gland architecture during pregnancy, as well as STAT5 activation, the expression of milk proteins and the ability to nurse pups successfully. This short form of the PRLR can bind to JAK2 and activate MAPK activity, but does not contain any PRLR docking sites. So the restoration of proliferation by this short form of PRLR during pregnancy triggered differentiation even when only half of the STAT5 docking sites were present. This concept is supported by the fact that mice with only one functional *Stat5* allele but with two functional *PrlR* alleles develop functional mammary tissue and can lactate[33].

Successful lactation depends on a pulsatile release of PRL from the pituitary gland. Galanin, a 29-amino-acid peptide, is a mitogen for lactotrophic cells, and mice with an inactivated *galanin* gene have reduced PRL levels during pregnancy, which causes an inability to nurse pups[34]. Lactation in these mice could be restored by PRL application[35], which further supports the hierarchy of these two hormones. In addition to regulating PRL concentration by controlling lactotrophic cells, galanin also influences the mammary epithelium directly. The pregnancy-induced alveolar architecture and milk-protein gene expression was not completely rescued in mice carrying two inactive *galanin* alleles[35]. However, similar to PRL, galanin induced the differentiation of mammary epithelium *in vitro*, as evidenced by the activation of STAT5.

It was originally proposed that PRL instructs the proliferation and differentiation of mammary epithelium through mechanisms that are specific to the PRLR in inducing luminal-cell-specific genes. However, experiments with hybrid receptors that contain the ligand-binding domain of the PRLR and the intracellular domain of the erythropoietin receptor (EPOR), which can recruit STAT5, have led to a revision of this model[36]. This hybrid receptor is activated by PRL and the signal, which is conveyed through the cytoplasmic domain of the EPOR and STAT5, is sufficient to restore the phenotype of PRLR-null mammary epithelium. Current evidence indicates that PRL — and probably other cytokines — represents a generic cue that activates transcriptional programmes that are shared between several cytokine receptors. Although these programmes might contain some cell-specific components, they seem to be of a general nature, mediating responses such as proliferation and cell survival. As discussed later, STAT5 and JAK2 are shared among many cytokine receptors and it seems that they convert the signal from a cell-specific receptor into a generic response.

Distinct roles of ERBB tyrosine kinase family members. Neuregulins, heregulins and other polypeptides that are related to EGF control vital cellular functions. Their effects are mediated by four distinct receptors from the ERBB family. The EGF receptor (ERBB1), ERBB2, ERBB3 and ERBB4 are unique receptor tyrosine kinases

PARACRINE
Describing, or relating to, a regulatory cell that secretes an agonist into intercellular spaces from which it diffuses to a target cell other than the one that produces it.

OSTEOCLAST
A mesenchymal cell that can differentiate into a bone-degrading cell.

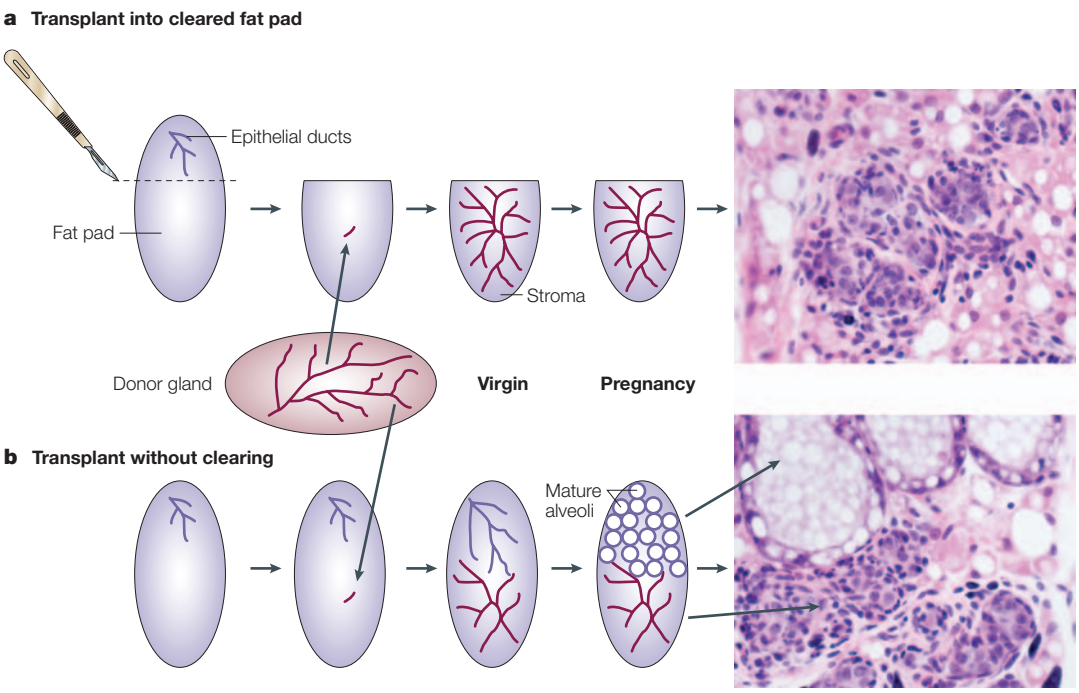
LACTOTROPHS
Prolactin-producing cells in the anterior lobe of the pituitary gland.

CYTOKINES
Initially identified as regulatory polypeptides secreted by immune cells, this family also contains non-immune molecules such as growth hormone and prolactin.

CORPUS LUTEUM
A temporary endocrine gland in the ovaries that produces progesterone.

HAPLO-INSUFFICIENCY
A state in which loss of only one of two alleles of a gene detectably disables its function.

Box 2 | **Mammary epithelial transplants**



Frequently, the deletion of a gene in the mouse influences the development and function of more than one organ. In particular, the absence of hormone receptors also affects ovarian function, which makes it difficult to distinguish between direct and indirect effects on mammary gland development. Furthermore, some mutants are not viable and so mammary epithelial development cannot be studied directly. These problems can be circumvented by transplanting mammary epithelial cells into a wild-type host[8,80]. In a 3-week-old mouse the epithelial ducts are still confined to the most proximal part of the fat pad near the nipple (see figure, part a). This area can be removed surgically to leave a 'cleared fat pad' into which epithelial cells from another (gene-deleted) animal can be transplanted. The transplanted epithelium (indicated in dark pink) can then develop in a wild-type stroma where it is exposed to the hormonal milieu of a normal animal. If the endogenous epithelium is left in place (see figure, part b), the mutant (dark pink) and wild-type (purple) epithelia will develop in the same fat pad under identical conditions, allowing side-by-side comparisons of both epithelia by '*in situ*' analyses such as histology, immunohistochemistry or *in situ* hybridization.

that can undergo homo- and heterodimerization, and activate several signalling pathways. An essential role for ERBB2 in a subset of human breast cancers has been recognized for more than 15 years[37], and the inhibition of ERBB2 signalling by the monoclonal antibody Herceptin to treat certain cases of metastatic breast cancers has highlighted its significance in cellular transformation[38]. A role for this family of tyrosine kinase receptors in normal mammary development and function has only recently emerged.

Both ERBB1 and ERBB4 are necessary for mammary development during pregnancy, although they exert their effects in different compartments. The first indication for a contribution of ERBB1 came from *waved-2* mice, in which *ErbB1* is mutated. These mice show impaired alveolar development[39]. For normal mammary development, the presence of ERBB1 within the stroma is required — an absence of ERBB1 from the epithelium does not abrogate pregnancy-mediated alveologenesis[31,40]. As ERBB1 can be activated by several ligands, the ablation of individual EGF-related hormones leads to no, or only partial, developmental defects, implying that there is considerable functional redundancy between these ligands[41].

Additional evidence that members of the ERBB family contribute to mammary function has come from transgenic mice expressing a DOMINANT-NEGATIVE form of ERBB2 (REF. 42). These mice fail to develop a functional alveolar compartment during pregnancy. As this protein lacks specificity and can form dimers with all ERBB-family members, the identity of the relevant ERBB2-dimerizing partner that was responsible for the phenotype could not be identified. Two lines of genetic evidence now point to ERBB4 as the molecule in question. Two research teams used different strategies to bypass the embryonic lethality of ERBB4-null mice[25,43] and derived similar conclusions. ERBB4-null foetuses die at embryonic day 11 (E11) owing to heart defects, and Golding and colleagues rescued this lethality with a transgene that targeted ERBB4 expression specifically to cardiac myocytes[43]. Rescued ERBB4-null mice were fertile but failed to nurse their litters. Histological

DOMINANT NEGATIVE
A defective protein that retains interaction capabilities and so competes with normal proteins, thereby impairing protein function.

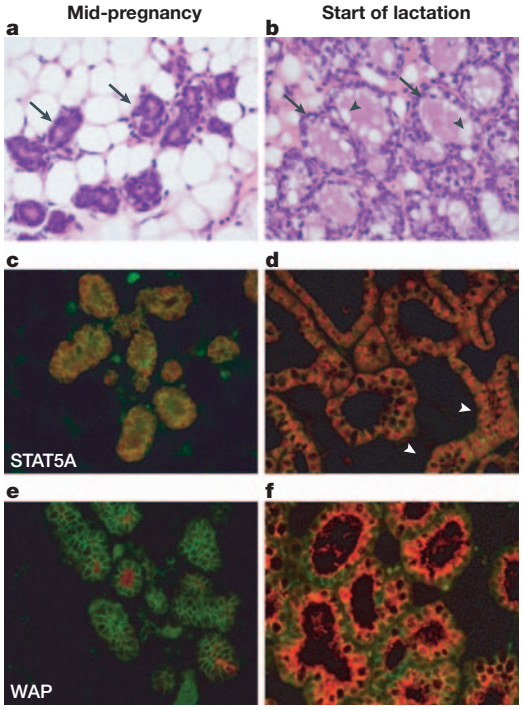


Figure 3 | **Progression of differentiation during pregnancy.** Several prognostic markers of mammary epithelial cell differentiation are acquired gradually in the course of pregnancy. At mid-pregnancy (left images) the gland consists of immature alveoli (arrows in part **a**) with a small lumen that is surrounded by cuboidal luminal cells. Moderate levels of STAT5A, the transcription factor that mediates signalling from the prolactin receptor (PRLR), are found in the majority of luminal cells (red staining in part **c**). In some of the alveoli, small amounts of the milk protein whey acidic protein (WAP) are made and secreted into the lumen (red staining in part **e**). At term, the alveoli are large (arrows in part **b**) and filled with milk, indicated by the pink staining material and lipid droplets (arrowhead in part **b**). Strong nuclear STAT5A staining (red stain in part **d**, arrowheads) reflects extensive signalling through the PRLR and ERBB4, and can be used as an indicator of active gene transcription. Large amounts of WAP (and other milk proteins) are synthesized and secreted into the lumen at the apical membrane (red stain in **f**). The cell adhesion molecule E-cadherin (green staining) outlines the luminal epithelial cells in parts **c**–**f**. STAT, signal transducer and activator of transcription.

analysis of mammary tissue at delivery revealed that the alveolar epithelium had undergone proliferation and expansion, and that its differentiation status, as measured by the expression of milk proteins, was within normal limits. However, in contrast to control tissue, lipid droplets accumulated within the luminal cells, which implied that these cells had failed to secrete milk efficiently, the hallmark of a fully differentiated cell. These studies could not distinguish between a direct effect of the absence of ERBB4 in mammary epithelial cells and an indirect, systemic effect. By deleting the ERBB4 gene specifically in mammary epithelium using CRE-*LOX*P-MEDIATED RECOMBINATION, Jones and colleagues resolved this issue[25]. Similar to the germline ERBB4 deletion, alveolar units in these mice failed to undergo complete functional differentiation and some reduction in alveolar proliferation and expansion was also observed. So the absence of ERBB4 in mammary epithelium has a direct effect on the cells. Like the PRLR, stimulation of ERBB4 activates STAT5 to convey growth and differentiation signals in the alveolar luminal cells. However, neither study could detect activated STAT5 in ERBB4-null alveolar epithelium at birth. This could indicate that ERBB4, through the activation of STAT5, has a more prominent role in the functional luminal cell during lactation than PRL does.

Interpreting multiple languages

JAK2 and STAT5. In evolutionary terms, STAT5 is an ancient transcription factor that responds to biochemical cues from a plethora of diverse cytokines, including growth hormone, erythropoietin, interleukins, PRL and ligands of the EGF family. Originally identified as a 'mammary gland factor' (MGF) that is activated by PRL and binds to promoter sequences in milk-protein genes[44], STAT5 has now taken centre stage in many cytokine signalling pathways[45]. STAT5A and STAT5B show 96% similarity at the amino-acid level and are encoded by two juxtaposed genes[46,47]. Mice from which *Stat5a* or *Stat5b* or both genes have been inactivated show biochemical alterations in many cell types. These alterations are consistent with those arising from inactivations or mutations of the respective activating cytokines and their receptors[45].

Inactivation of *Stat5a* in mice caused no overt phenotype, except for the failure to lactate[48], suggesting a partial compensation through STAT5B. Proliferation and expansion of the alveolar compartment was only slightly reduced but the luminal cells failed to undergo functional differentiation to produce milk. Analysis of mammary tissue devoid of both STAT5A and STAT5B[49] has confirmed that these two transcription factors display partially redundant functions in mammary development[50]. As mice in which both STAT5 genes had been targeted were infertile owing to non-functional corpora lutea[49] — similar to the PRLR-null mice[29] — pregnancy-mediated mammary development therefore had to be investigated in tissue from STAT5A/B-null mice that was transplanted into immunocompromised, but otherwise wild-type, mice (BOX 2). The complete absence of lobuloalveolar development, probably as a result of the lack of proliferation, was comparable to that seen in the absence of PRLR or JAK2 (REFS 13,29,31,50,51). The use of mice in which the entire *Stat5* locus was flanked by *lox*P sites provided further insight into the multiple roles of STAT5 throughout pregnancy[33]. As expected, Cre-mediated inactivation of *Stat5* in MAMMARY ALVEOLAR progenitor cells resulted in the same physiological consequences that were observed when the gene was ablated in the germline. By contrast, loss of *Stat5* late in pregnancy, after the epithelium has initiated the maturation programme, led to premature cell death, indicating a role for STAT5 in cell survival. STAT5 has now emerged as a crucial switch not only for the proliferation and differentiation of mammary luminal

CRE-*LOX*P-MEDIATED RECOMBINATION
A tool for cell-specific gene deletion. It uses Cre recombinase from bacteriophage P1, which mediates intra- and intermolecular site-specific recombination between *lox*P sites.

MAMMARY ALVEOLUS
The functional unit in the mammary gland that produces milk.

cells, but also as a regulator of cell survival and function during lactation.

Although the individual *Stat5* knockouts show distinct phenotypes, this does not mean that STAT5A and STAT5B have inherently different properties — instead it reflects differences in the expression patterns of the two genes. For example, STAT5A is the predominant isoform in mammary tissue and STAT5A-, but not STAT5B-, null mice show blunted mammary development. By contrast, STAT5B is the predominant isoform in liver and so STAT5B-null mice show defects in this tissue[52]. STAT5 can be activated in the mammary epithelium through the phosphorylation of a specific tyrosine residue by JAK2 and ERBB4. JAK2 seems to be the crucial activator of STAT5, at least during the early stages of pregnancy, as evidenced by the mammary phenotype of JAK2-null mammary epithelium[51,53,54].

The concept that PRL and placental lactogens are the key hormones that activate STAT5 and thereby induce a developmental programme leading to the production of milk-secreting cells was firmly established until the finding that ERBB4 also conveys its information through STAT5. An intriguing interpretation would be that both PRLR and ERBB4 control distinct and possibly overlapping features of alveolar development. Clearly, the PRLR is absolutely essential for alveolar proliferation in early pregnancy, whereas the loss of ERBB4 affects the functional differentiation of luminal cells after the proliferative phase. Yet only the specific inactivation of the PRLR in differentiated luminal cells will address its relevance during established lactation.

Modulating the flow of information

The almost identical nature of mammary defects after deletion of *PrlR*, *Jak2* or *Stat5* illustrates the importance of this cascade in luminal cell proliferation and differentiation. Although it is known how hormones activate this pathway, the nature of the 'brakes' that allow modulation and cessation of these signals has so far been an enigma. Two main components and distinct mechanisms to avoid either precocious or sustained JAK–STAT signalling during pregnancy have now been discovered. These are mediated by members of the SOCS family of proteins and, unexpectedly, caveolin-1 (CAV1), a structural component of lipid rafts.

SOCS members as brakes. The eight members of the SOCS family (SOCS1–7 and cytokine-inducible Src-homology-2 (SH2) protein (CIS)) interact with JAKs and cytokine receptors to curtail the activation of STAT proteins[55]. SOCS1, which is linked to the modulation of interferon signalling in the immune system, has also been shown genetically to be an essential regulator of PRL signalling in the mammary epithelium during pregnancy[56]. *Socs1*-null mice have immunological defects, but these can be overcome by simultaneously deleting *Ifn1* genes. Such mice showed excessive alveolar proliferation during pregnancy and elevated STAT5 activity during lactation, implying that SOCS1 inhibits PRL signalling. As mentioned above, luminal cell proliferation and differentiation during the first pregnancy is incomplete in the presence of only one *PrlR* allele. An equivalent loss of one *SOCS1* allele in PRLR hemizygous mice restored functional alveolar development and STAT5 activity, which elegantly shows the role of SOCS1 in modulating PRL signals.

In vitro studies have shown that SOCS molecules bind to tyrosine residues in cytokine receptors and block the binding and activation of STAT molecules. Expression of SOCS3 is controlled by STAT5 and its levels are induced during pregnancy[57], which indicates that it is part of a negative-feedback loop. SOCS3 binds to tyrosine residues in the gp130 receptor, which is the shared subunit of receptors for cytokines such as interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF). Activation of gp130 signalling in the mammary epithelium occurs during involution and leads to the activation of STAT3, the presence of which is required for involution[58–61]. Therefore, it can be predicted that SOCS3 has a role in modulating gp130-controlled remodelling during involution. *Socs3*-null embryos die because of a placental defect at mid-pregnancy (~12 days of embryonic development)[62,63], at which point it is too early to rescue the mammary epithelium by transplantation. But inactivation of *Socs3* specifically in mammary epithelium using Cre-*lox*P-mediated recombination established a role for SOCS3 in the remodelling of mammary tissue during involution (M. Pacher-Zavisin, G.W.R. and L.H., unpublished observations), indicating that it is part of the cytokine signalling pathway that is activated by the gp130 receptor[60].

CAV1. The discovery that CAV1 is required for controlling STAT5 activity provided an unexpected twist to cytokine signalling[64]. CAV1 is an essential structural component of CAVEOLAE. The loss of both *Cav1* alleles results in precocious mammary gland development during pregnancy and concomitant precocious activation of STAT5. Molecular analyses have established that CAV1 abrogates PRL-induced gene expression by sequestering JAK2, which therefore cannot activate STAT5. These studies highlight that the compartmentalization of components within the cell controls their availability on cytokine stimulation.

Executing STAT5 signals

Inactivation of the genes encoding PRLR, ERBB4, JAK2 and STAT5 has highlighted a role for this pathway in the proliferation, survival and differentiation of mammary epithelium. Implementing these programmes is expected to involve several proteins, and the removal of a single STAT5 target gene will not recapitulate the lesions resulting from the absence of STAT5. Several types of STAT5 target gene have been identified so far (FIG. 4): genes that encode proteins that merely reflect the differentiation status of the cell; those encoding proteins that promote cell proliferation and control cell survival; a gene encoding a growth factor and one encoding a transcription factor.

CAVEOLAE
Specialized domains in the plasma membrane that form small invaginations and are involved in vesicular trafficking and cell signalling.

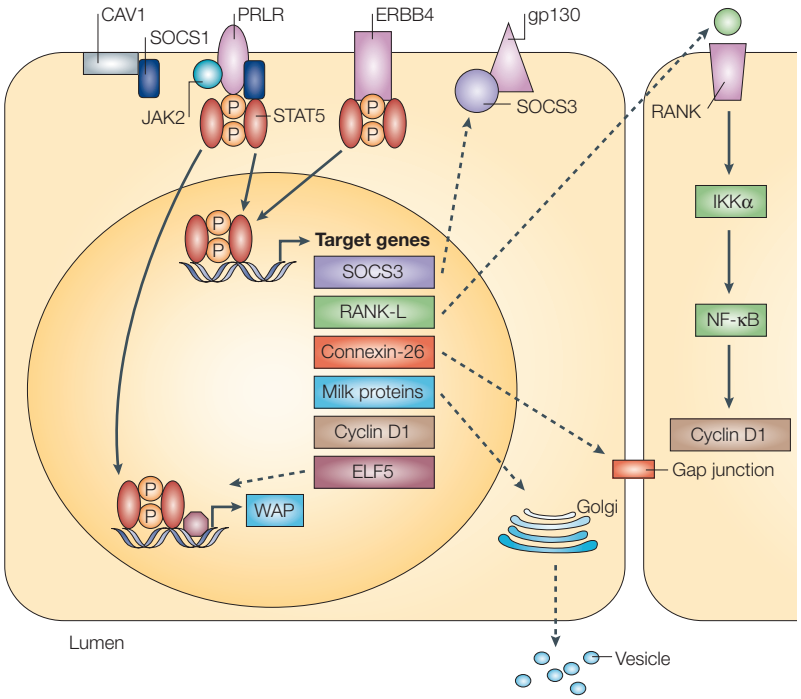


Figure 4 | **Processing of cytokine information during pregnancy.** Cytokine signalling through the prolactin receptor (PRLR) and ERBB4 activates signal transducer and activator of transcription-5 (STAT5). On PRL binding, the conformation of the PRLR dimer changes and the associated Janus kinase JAK2 phosphorylates PRLR on specific tyrosine residues. When STAT5 binds to these residues, JAK2 phosphorylates one tyrosine residue on STAT5, inducing its dimerization and nuclear translocation. Suppressor of cytokine signalling-1 (SOCS1) binds to PRLR and negatively regulates STAT5 signals. STAT5 dimers bind to specific sites in the promoters of target genes and induce their transcription. Bona fide target genes include those encoding SOCS3, RANK-L (receptor activator of nuclear factor κB (NF-κB)-ligand), connexin-26, milk proteins, cyclin D1 and the transcription factor ELF5. SOCS3 negatively regulates cytokine signalling through gp130-containing receptors, which are active mainly during involution[60]. RANK-L is secreted and activates the RANK–NF-κB pathway (right-hand cell); connexin-26 is a component of gap junctions; and cyclin D1 promotes mammary epithelium proliferation. ELF5 is a transcription factor that, together with STAT5, induces transcription of whey acidic protein, a prominent milk component. STAT5 itself activates the transcription of several milk-protein genes. Milk components, including micelles, are assembled in the Golgi apparatus, and vesicles (blue circles) are secreted into the alveolar lumen. JAK2 can also associate with caveolin-1 (CAV1). IKKα, inhibitor of NF-κB (IκB) kinase-α; P, phosphate.

RANK-L, RANK, cyclin D1 and IGF-2. The RANK-L gene, which encodes an osteoclast differentiation factor from the TNF family, is under the control of PRL itself[65] and is a bona fide STAT5 target. This indicates that PRL, a growth factor by itself, can activate production of another growth factor. A role for RANK-L and its receptor RANK in mammary gland development was established in mice from which either of the two genes was deleted[24]. These mice were unable to nurse their young because of an inhibition of alveolar development during pregnancy. In mammary epithelial cells from these mice, activation of the anti-apoptotic molecule AKT/protein kinase B (PKB) was reduced, alveolar cell death during pregnancy was increased and cell proliferation was reduced. However, ALVEOLAR BUDS still formed, which implied that RANK-L is required for later steps of alveolar development, such as differentiation. One of the downstream events in RANK signalling is the activation of NF-κB. The existence of this link in the mammary gland was established by mutating IκB kinase-α (IKKα), a subunit of inhibitor of NF-κB (IκB)[66]. Mutations of serine residues in the ACTIVATION LOOP rendered IKKα inactive and severely impaired NF-κB activity. Mammary epithelium in these mice expanded during pregnancy but was unable to functionally differentiate[53,66]. The concomitant reduction in cyclin D1 expression observed during pregnancy in this mutant identified this cell-cycle regulator as a crucial downstream target of NF-κB signalling. This was supported by evidence that expression of a cyclin D1 transgene rescued epithelial differentiation and the lactation defect in these mice[53,66].

The cyclin D1 gene is activated not only by the RANK–NF-κB pathway but also by STAT5 directly through a γ-IFN-activated site (GAS) within the promoter. Loss of cyclin D1 leads to a paucity of alveolar cells, which also fail to functionally differentiate[67,68].

Insulin-like growth factor-2 (IGF-2) has been proposed as another mediator of PRL signalling[69]. Its expression can be induced in primary mammary cells by PRL, and ectopic expression of IGF-2 can partially rescue pregnancy-mediated expansion of PRLR-null mammary epithelial cells[69]. IGF-2-null mice can nurse their pups, which shows that this growth factor is not essential for normal mammary development *per se*. However, a transient mammary epithelial growth defect in mid-pregnancy has been observed, which was alleviated at term, indicating that IGF-2 might have a modulatory role during a specific time window.

Junctional integrity. The establishment of functional alveoli depends on the polarization of the luminal cells and the formation of junctions between them. The gene encoding connexin-26 (*Cx26*), an essential integral component of gap junctions, is a direct target gene of STAT5 (REF. 70). It is expressed during pregnancy and its loss is not compensated for by other connexins. Its essential role in mammary development has been shown in cell-specific knockout mice[71]. Specific deletion of *Cx26* in the mammary epithelium led to a high level of cell death, causing a failure to nurse pups. Like *Cx26*, the *Cx32* gene is also controlled by STAT5, but its expression is confined to the lactation stage. Loss of *Cx32* did not alter mammary function, which can be explained by a compensatory function of connexin-26 (REF. 70).

Milk proteins. The genes for milk proteins, the expression of which defines a mature and differentiated mammary luminal cell, are the classic targets of STAT5 in the mammary epithelium, and their transcriptional stimulation during pregnancy is mediated by GAS sequences within their promoter regions[72,73]. In the absence of both STAT5A and STAT5B, none of the milk proteins, including whey acidic protein (WAP) and β-casein, is expressed[50]. A considerable reduction can be observed in the absence of only STAT5A[48].

ALVEOLAR BUD
A small evagination from a main duct that forms during the oestrous cycle and at the initiation of pregnancy.

ACTIVATION LOOP
A conserved structural motif in kinase domains that needs to be phosphorylated for full activation of the kinase.

Box 3 | **Key proteins during pregnancy**

The following proteins control the proliferation, differentiation, survival and function of mammary secretory epithelial cells during pregnancy.

Hormones
- **Progesterone: steroid hormone synthesized by the corpus luteum.**
- **Prolactin (PRL): peptide hormone produced preferentially in lactotrophic cells in the pituitary gland but also in mammary epithelial cells.**
- **EGF family: a class of growth factors structurally related to epidermal growth factor.**
- **Receptor activator of nuclear factor κB (NF-κB)-ligand (RANK-L): osteoclast differentiation factor from the tumour necrosis factor (TNF) family.**

Receptors
- **Prolactin receptor (PRLR): the PRLR is activated by the peptide hormones prolactin (PRL) and placental lactogens.**
- **ERBB4: member of the EGF (ERBB) receptor family. ERBB4 is a tyrosine kinase and forms homodimers and heterodimers with other members of the ERBB family.**
- **RANK: receptor activator of NF-κB.**

Kinases
- **JAK2: Janus kinase (JAK) 2 is a tyrosine kinase associated with type 1 cytokine receptors, including the PRLR, growth hormone receptor and the erythropoietin receptor. It phosphorylates specific tyrosine residues on itself, the receptors, and on STAT3 and STAT5.**

Transcription factors
- **STAT5: member of the family of signal transducers and activators of transcription.**
- **ELF5: member of the ETS family.**

Cell-cycle regulators
- **Cyclin D1.**

Structural proteins
- **Connexin-26 and connexin-32: members of the connexin family of proteins that form gap junctions.**

Milk proteins
- **WAP: the whey acidic protein (WAP) is a prominent protein in mice and its transcription is controlled to a large extent by STAT5, which binds in the promoter region. In addition, an ETS site (possibly recognized by ELF5) in the *WAP* gene promoter is necessary for the temporal regulation of *WAP* expression during pregnancy.**

Survival pathways. Little is known about the pathways that convey luminal cell survival. Among the members of the BCL2 family, Bcl-X is by far the most prominently expressed in the mammary epithelium and its gene is induced during pregnancy[74]. In several *in vitro* systems, *Bcl-X* is controlled by STAT5 through a GAS motif in the promoter[75]. However, inactivation of *Bcl-X* specifically in mammary epithelium did not abrogate luminal cell survival during pregnancy, but instead resulted in accelerated remodelling after cessation of lactation[74].

ELF5. DNA microarray analyses have identified ELF5 as a protein that is preferentially expressed in mammary tissue and the expression of which is strongly induced during pregnancy[76] — this implies that it could be under the control of PRL signalling[70]. ELF5 is a member of the ETS FAMILY of transcription factors and its expression is specific to epithelial cells of EXOCRINE GLANDS[35,77]. *Elf5*-null foetuses die at embryonic day 7.5, which points to a crucial function during embryogenesis. Mice that have only one functional *Elf5* allele showed abrogated pregnancy-mediated alveologenesis[78]. Notably, the lesions were similar to those observed in the absence of PRLR[29], STAT5 (REFS 33,50) or JAK2 (REFS 51,54). Alveolar buds formed but failed to proliferate and mature, as indicated by the absence of milk proteins. Although circumstantial, this points to the *Elf5* gene being a bona fide STAT5 target. It is intriguing that STAT5 as a transcription factor not only activates genes that control specific features of epithelial cells, such as cell proliferation and differentiation, but also a gene that encodes another transcription factor, which itself could control differentiation programmes. ETS transcription factors modulate the expression of the milk protein gene *WAP* during pregnancy, but not during lactation, as shown in transgenic mice[79], and it can be postulated that they activate other genes in mammary epithelium in synergy with STAT5.

BCL2
An anti-apoptotic protein. It is the founding member of the BCL2 family of pro- and anti-apoptotic proteins.

ETS FAMILY
Proto-oncogene family related to v-*ets*, one of the oncogenes of the acutely transforming avian erythroblastosis virus E26.

EXOCRINE GLAND
A gland of epithelial origin that secretes (directly or through a duct) onto an epithelial surface.

FORWARD GENETICS
A genetic analysis that proceeds from phenotype to genotype by positional cloning or candidate-gene analysis.

Conclusions

A picture is emerging of how peptide hormones control the genesis of a functional mammary gland during pregnancy (FIG. 4; BOX 3). It is now firmly established that, on ligand binding, the receptors convey their signals through tyrosine kinases and the transcription factor STAT5. The timing, intensity

and duration of these signals are modulated by SOCS proteins and caveolin. The genes activated by STAT5 encode proteins that control cell proliferation, gap junction components, growth factors, members of the SOCS family, milk proteins and at least one other transcription factor, which, in conjunction with STAT5, can activate the transcription of milk-protein genes. Experiments from many laboratories have identified additional genes that are either activated or suppressed during pregnancy. The challenge ahead is to place these genes and the proteins they encode into the larger picture of mammary development and function. Although essential, traditional mouse genetics will not be the only pillar for the understanding of the physiological significance of each protein in the puzzle of mammary gland development. It will be necessary to introduce *in vitro* approaches that use primary cells or cell lines combined with FORWARD GENETICS. Only when it is possible to rescue the absence of a gene in the mammary epithelium by introducing a gene encoding a downstream target will it be possible to define their position in the signalling network. Towards this goal, it will be necessary to develop primary cells that can form a functional epithelium *in vivo* after being genetically manipulated *in vitro*.

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Competing interests statement

The authors declare no competing financial interests.

Online links

DATABASES

The following terms in this article are linked online to:

Swiss-Prot: http://www.expasy.ch/sprot

Bcl-X | CAV1 | *Cx26* | *Cx32* | ELF5 | ERBB1 | ERBB2 | ERBB4 | gp130 | JAK2 | PRL | PRLR | RANK | RANK-L | SOCS1 | SOCS3 | STAT3 | STAT5A | STAT5B

FURTHER INFORMATION

Lothar Hennighausen's laboratory:
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